Technical Data Sheet

Violet Fluorescent Cell Barcoding Dyes for 561570

Product Information

Material Number: 561571
Size: 800 tests
Reactivity: QC Testing: Human
Component: 51-9007169
Description: Cell Barcoding Dye 500
Component: 51-9007170
Description: Cell Barcoding Dye 450

Description

Components
51-9007170 Cell Barcoding Dye 450 (CBD450)
51-9007169 Cell Barcoding Dye 500 (CBD500)
561550 Fluorescent Cell Barcoding Wash Buffer (Four 500 mL Bottles)

One of the following phosphosite-specific fluorescent antibodies:
612593 Alexa Fluor® 647 Mouse Anti-ERK1/2 (pT202/pY204) 50 Tests
612595 Alexa Fluor® 647 Mouse Anti-p38 MAPK (pT180/pY182) 50 Tests
612597 Alexa Fluor® 647 Mouse Anti-Stat1 (pY701) 50 Tests
557815 Alexa Fluor® 647 Mouse Anti-Stat3 (pY705) 50 Tests
612599 Alexa Fluor® 647 Mouse Anti-Stat5 (pY694) 50 Tests
612601 Alexa Fluor® 647 Mouse Anti-Stat6 (pY641) 50 Tests

BD Phosflow™ Violet Fluorescent Cell Barcoding Kit

800 tests is achieved by multiplexing up to 16 separate samples for each single antibody staining. Each kit provides antibody reagent sufficient for up to 50 single antibody stainings.

Fluorescent Cell Barcoding is a technique that facilitates high-throughput multiparameter flow cytometry applications by allowing multiple target cell samples to be combined into a single tube for multicolor staining and analysis. Each cell sample is “barcoded,” using one or two covalently bound fluorescent dyes-each dye is used at up to three specific concentrations. This allows the cell samples to be distinguished by multicolor flow cytometry based on their distinct fluorescence emission wavelengths and intensities. Multiplexed barcoded cell samples can further be stained with fluorescent antibodies that are specific for CD antigens and intracellular phosphorylated signaling proteins. Thus, Fluorescent Cell Barcoding enables a high-throughput approach to determine the signaling profiles of leukocyte subsets. Analyses that could benefit from this barcoding technique include (but are not limited to) multi-donor, dose-response and/or time-course studies for cellular response modifiers (eg, drugs, cytokines, polyclonal activators, adjuvants).

The BD Phosflow™ Violet Fluorescent Cell Barcoding Kit provides reagents for successfully barcoding T cell subsets from human whole blood samples for the purpose of studying phosphorylated protein induction in the MAP Kinase and JAK/STAT signaling pathways. The kit includes two fluorescent dyes, Cell Barcoding Dye 450 (CBD450) and Cell Barcoding Dye 500 (CBD500), Fluorescent Cell Barcoding Wash Buffer, and one phosphosite-specific antibody for barcoding and immunostaining fixed and permeabilized cell samples. The barcoding dyes are used at various concentrations either separately, or in combination to barcode up to sixteen different cell samples. The Kit provides a choice of an Alexa Fluor® 647-conjugated, phosphosite-specific antibody that can be used for analysing intracellular signaling events. The following phospho-specific antibodies are available to choose from: ERK1/2 (pT202/pY204); p38 MAPK (pT180/pY182); Stat1 (pY701); Stat3 (pY705); Stat5 (pY694); or Stat6 (pY641). Each of these antibodies has been shown to stain barcoded fixed and permeabilized human peripheral blood lymphocytes.

Fluorescent antibodies specific for T-cell associated surface markers have been validated for use with the Violet Fluorescent Cell Barcoding Kit using the fixation and permeabilization procedure specified in this kit. The Lyse/Fix and Permeabilization reagents must be ordered separately. Cell samples prepared using the reagents provided in, or specified by, this kit can be analyzed using the BD FACSCanto™ II, BD LSRFortessa™ or BD™ LSR II Flow Cytometer Systems.
Fluorescent Cell Barcoding Workflow Overview

**Prepare Barcoding Dyes in 96 Well Plate:**
- Add 200 µL of DMSO to well A1. Add 100 µL of dye solutions to wells with low (L), medium (M) or high (H) concentrations of CBD450 (450) dye as shown.
- Add 100 µL of CBD500 (500) dye solutions to wells as shown.

**Preferred Assay Procedure:**
**Plate 1**
- Transfer 20 µL from A1–A4 wells (CBD Dye Dilution 96 Well Plate) to appropriate A1–A4, B1–B4, C1–C4, and D1–D4 wells (Fluorescence Cell Barcoding Deep Well Plate).
- Transfer 20 µL of CBD450 High and CBD500 High from Stock Tubes to A5 and B5 wells (Barcoding Deep Well Plate).

**Plate 2**
- Prepare Cells in Deep Well Plate:
  - Cells
  - Fix cells
  - Permeabilize cells
  - Resuspend cells in 500 µL of 50% Perm Buffer III

**Fluorescence Cell Barcoding Deep Well Plate**

**Plate 3**
- Harvest Unlabeled Cells to be stained (E1–E5) with fluorescent Abs for fluorescence compensation.
- Combined Barcoded Cells Cells from wells A1 through D4 are combined into reservoir with Wash Buffer. Mix cells together & transfer to a flow tube labeled “Combined”.
- Collect Unlabeled Cells (C5 or D5) and 450 H and 500 H Cells labeled with high concentrations of CBD450 (well A5) or CBD500 (well B5) for fluorescence compensation.

**Application Notes**

**Application**

| Intracellular staining (flow cytometry) | Routinely Tested |

**Recommended Assay Procedure:**

Please see next page.
Required equipment
• BD FACSCanto™ II, BD LSRFortessa™ or BD™ LSR II Flow Cytometer
• BD Falcon™ Round-Bottom 12 × 75-mm Polystyrene Tubes (Cat. No. 352008)
• Multichannel Pipet (100-1200 μL capacity; Rainin Multi 1200 12 channel, Cat. No. E12-1200 or equivalent)
• Multichannel Pipet (20-200 μL capacity)
• Benchtop Aspirator
• Aspirating Manifold (V&P Scientific; Cat. No. VP 187A)
• Centrifuge with 96 Deep Well Plate Holder
• 37°C Incubator
• 37°C Water Bath

Materials required but not provided
• Fresh EDTA or Heparin anticoagulated whole blood
• BD Phosflow™ Lyse/Fix Buffer 5× (Cat. No. 558049). This buffer needs to be diluted to 1× (ie, 1 volume 5× buffer + 4 volumes distilled water) and pre-warmed to 37°C immediately prior to use.
• BD Phosflow™ Perm Buffer III (Cat. No. 558050) (Stored at -20°C)
  • 50% BD Phosflow™ Perm Buffer III (Dilute with PBS; store at -20°C)
• Distilled Water
• 1× PBS (Room Temperature)
• Bovine Serum Albumin (BSA) (Sigma; Cat. No. A7906)
• Dimethyl Sulfoxide (Sigma; Cat. No. D2650), anhydrous, fresh
• Eppendorf® microcentrifuge tubes or 2 mL cryovials
• 2.2 mL Storage Plate, MARK II (96 Deep Well; Abgene Cat. No. AB0932)
• Adhesive 96 Well Plate Sealers for 96-well plates (Costar; Cat. No. 3095 or equivalent)
• Reagent Reservoirs (VWR; Cat. No. 89094-676)

Note: EDTA-anticoagulated whole blood preserves better cellular light scattering properties than heparinized blood when Phorbol 12-Myristate 13-Acetate (PMA) is used for cellular activation.

Fluorescent antibodies for the multicolor staining of T cell subpopulations
The following fluorescent antibodies specific for CD markers have been validated for staining T cell subpopulations under these Fluorescent Cell Barcoding conditions. These antibodies can also be used to stain cells as controls for fluorescence compensation.

• CD3 (UCHT1)-PE (Cat. No. 555333)
• CD4 (SK3)-PerCP-Cy™5.5 (Cat. No. 341654)
• CD8 (RPA-T8)-Alexa Fluor® 488 (Cat. No. 557704)
• CD4-(RPA-T4)-Alexa Fluor® 647 (Cat. No. 557707) - Cannot be used with phosphosite-specific Abs.

Procedure

Note: Please read the entire procedure carefully and review the Fluorescent Cell Barcoding Workflow Overview (Figure 1) before you begin.

I. Reagent preparation

Preparation of CBD450 and CBD500 dye solutions in DMSO

A. Preparation of Stock CBD450 and CBD500 dye solutions
1. Label six 15 mL conical tubes as: CBD450 High, CBD450 Medium, CBD450 Low, CBD500 High, CBD500 Medium, and CBD500 Low.
2. Add 1 mL DMSO to the vials containing CBD450 and CBD500 dye provided in the kit. Mix thoroughly by vortexing to ensure the dyes are completely dissolved.
3. Microfuge the vials briefly (2-5 seconds) and transfer each 1 mL dye solution to the appropriately labeled CBD450 High or CBD500 High 15 mL conical tube.
4. Add an additional 5.82 mL DMSO to each CBD450 High and CBD500 High labeled tubes and mix well by vortexing. The total dye solution volume in each tube will be 6.82 mL.
5. Add 4.5 mL of DMSO to the CBD450 Medium and CBD500 Medium labeled tubes and transfer 1.5 mL of CBD450 High and CBD500 High solutions to the appropriate CBD450 Medium and CBD500 Medium labeled tubes. Mix well by vortexing. The total dye volume in each tube is 6 mL.
6. Add 4 mL of DMSO to the CBD450 Low and CBD500 Low labeled tubes and transfer 1 mL of CBD450 Medium and CBD500 Medium solutions to the appropriate CBD450 Low and CBD500 Low labeled tubes. Mix well by vortexing. The total dye volume in each tube is 5 mL.
7. The following tubes are now ready for use (see Table 1):
Table 1. CBD450 and CBD500 Dye solutions

<table>
<thead>
<tr>
<th>Tube Name</th>
<th>Final Volume</th>
<th>Tube Name</th>
<th>Final Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBD450 High</td>
<td>5.32 mL</td>
<td>CBD500 High</td>
<td>5.32 mL</td>
</tr>
<tr>
<td>CBD450 Medium</td>
<td>5.00 mL</td>
<td>CBD500 Medium</td>
<td>5.00 mL</td>
</tr>
<tr>
<td>CBD450 Low</td>
<td>5.00 mL</td>
<td>CBD500 Low</td>
<td>5.00 mL</td>
</tr>
</tbody>
</table>

Note: Do not mix the dyes at different concentrations.

8. Aliquot 1 mL (or user-defined volume) each of the above dye solutions to appropriately labeled Eppendorf® microcentrifuge tubes or 2 mL cryovials (the tube labels should include the name of the dye, concentration, and date).

9. Freeze all dye aliquots at -80°C protected from light.

Note: The stock dye solutions can be stored at -80°C for up to 3 months. Aliquots can be thawed and refrozen up to 10 times without significant reduction of fluorescence intensities of barcoded cell samples. Determine the volume and number of stock dye solution aliquots based on this information.

B. Reagent preparation-Additional instructions

1. Thaw aliquots of stock dyes.
   a. Depending on the volume, aliquots may take 30 min or longer to fully thaw and equilibrate to ambient temperature especially when thawed at room temperature.
   b. The stock dye solutions can be thawed in a 37°C dry bath (such as a heat block with accurate temperature control) which will shorten the time needed to thaw the dye solutions. Heat until just thawed (no longer than 20 minutes).
   c. It is critical to fully equilibrate the dye solutions to ambient temperature before opening the tubes. This prevents condensation from forming and being absorbed into the dye solution. The barcoding dyes may become unstable during prolonged exposure to aqueous solutions.

2. Store BD Phosflow™ Perm Buffer III at -20°C. Prepare 50% Perm Buffer III by diluting 1:2 (ie, 1 volume of Perm Buffer III plus 1 volume of PBS). Prepare 15 mL of the 50% solution for each 4 × 4 barcode matrix or prepare stock in advance. Both 1× Perm Buffer III and the 50% Perm Buffer III solution are required for each experiment.

3. Prepare 1× BD Phosflow™ Lyse/Fix Buffer from the 5× stock solution. 1.2 mL of 1× Lyse/Fix Buffer is required for each sample per each deep well. Prepare 10-20% fold excess of the buffer than required.
   a. Prewarm 1× Lyse/Fix Buffer for 15 to 60 min at 37ºC prior to use. Discard any unused pre-warmed buffer.

Note: 1× Lyse/Fix Buffer that has not been prewarmed, can be stored for 24 hrs at RT when protected from light.

4. Dilute Barcoding Wash Buffer 1:4 in 1× PBS (ie, 1 volume of Wash Buffer plus 3 parts PBS = 1× Barcoding Wash Buffer). Prepare about 150 mL for each 4 × 4 barcode experiment. Prepare this buffer fresh daily and use at RT.

5. Dilute each activator to the desired concentration.
   a. Dilute cytokines and PMA in 1× PBS containing at least 1 mg/mL bovine serum albumin (BSA) to help stabilize them.
   b. Dilute activators just prior to use and keep the diluted activators on ice until use.
   c. See Table 2 for a suggested panel of activators.
   d. Plan to add no more than 20 μL of activator to the appropriate wells. This ensures a final blood plus activator volume of no greater than 120 μL.

Table 2. Human experimental model systems that have been shown to work

<table>
<thead>
<tr>
<th>Activator</th>
<th>Vendor</th>
<th>Cat. No.</th>
<th>Final Concentrations</th>
<th>Temp</th>
<th>Time</th>
<th>Induced Target Phosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>hIL-2</td>
<td>BD</td>
<td>554603</td>
<td>100, 10, 1 ng/ml</td>
<td>37°C</td>
<td>15 min</td>
<td>Stat5</td>
</tr>
<tr>
<td>hIL-4</td>
<td>BD</td>
<td>554605</td>
<td>100, 10, 1 ng/ml</td>
<td>37°C</td>
<td>15 min</td>
<td>Stat6</td>
</tr>
<tr>
<td>hIL-6</td>
<td>BD</td>
<td>550071</td>
<td>100, 10, 1 ng/ml</td>
<td>37°C</td>
<td>15 min</td>
<td>Stat3</td>
</tr>
<tr>
<td>PMA</td>
<td>Sigma</td>
<td>P8139</td>
<td>400, 200, 100 nM</td>
<td>37°C</td>
<td>15 min</td>
<td>ERK1/2, p38 MAPK</td>
</tr>
<tr>
<td>hIFN-α</td>
<td>Sigma</td>
<td>I-4276</td>
<td>40,000, 4,000, 400 units/ml</td>
<td>37°C</td>
<td>15 min</td>
<td>Stat1, (Stat3, Stat5 weaker)</td>
</tr>
</tbody>
</table>
II. Experimentation

Note: Please refer to Figure 1, Fluorescent Cell Barcoding Workflow Overview to facilitate comprehension of the Experimentation

A. Cellular activation

1. Aliquot 100 μL of fresh whole blood to required wells of a deep-well plate (see Figure 1 for the plate layout). This can be done using either a single channel or multichannel pipet with a 100 or 200 μL capacity.
   a. For a 6-color experiment (two barcoding dye colors plus four different fluorescent antibody colors), 7 additional cell samples (in wells) will be needed for fluorescence compensation.
   b. For barcoding 16 different cell samples, we recommend aliquoting blood to 25 wells of a 96-well deep well plate in a 5 × 5 grid format (Figure 1, Cell Preparation Deep Well Plate). This accounts for a 4 × 4 grid pattern of barcoded cell samples and an additional 9 wells for cellular controls.
   c. Add 20 µL of activator to desired wells.

2. Mix the well contents by vortexing the plate gently. Incubate the plate in a 37°C incubator for 15 minutes, or other desired time period.

B. Lyse red blood cells and fix leukocytes

Note: Longer incubation times in Lyse/Fix Buffer may result in decreased surface marker staining.

3. Using a multichannel pipet, add 1.2 mL of 1× Lyse/Fix Buffer (pre-warmed to 37°C) to ALL of the wells containing blood samples. Mix well contents thoroughly by pipetting up and down 3 times.
   a. We recommend using a multichannel pipet or a robotic device to efficiently add and mix Lyse/Fix Buffer with cell samples.
   b. If using an adjustable pipet, set the volume to 900 μL for mixing to avoid contaminating the pipet and cell samples.

4. Seal the plate with a plate sealer and incubate in a 37°C incubator for 15 min.

5. Centrifuge the cells at 600 g for 5 min. Remove supernatants by aspirating with a manifold aspirator. The residual volume after aspiration should be no greater than 50 μL per well.

6. Vortex the plate vigorously to loosen the cell pellets.

7. Wash once by adding 1 mL of 1× PBS to each well using a multichannel pipet.

8. Centrifuge the cells at 600 g for 5 min. Remove supernatant by aspirating with a manifold. The residual volume after aspiration should be no greater than 50 μL per well.

C. Cellular permeabilization

Note: Unless otherwise instructed, carry out all of the cellular permeabilization steps on ice. Minimize unnecessary exposure of cells to Perm Buffer III especially at temperatures above 4°C. Prolonged exposure of cells to Perm Buffer III may compromise the staining of certain cellular target antigens.

9. Vortex the plate vigorously to loosen the cell pellets and place the plate containing the cells on ice. Add 1 mL of cold 1× Perm Buffer III to each well using a multichannel pipet. Mix the well contents by pipetting up and down 3 times. Seal the plate with a plate sealer.

10. Incubate the plate on ice for 30 min.

11. Immediately after the 30 min incubation, centrifuge the plate at 600 g for 5 min. Immediately remove the supernatant by aspirating with a manifold. The residual volume after aspiration should be no greater than 50 μL per well.

12. Vortex the plate vigorously to loosen cell pellets. Place the plate on ice. Add 500 μL of cold 50% Perm Buffer III to each well. Mix the well contents by pipetting up and down 3 times or by gentle vortexing. The samples are now ready to be barcoded (step 16).

D. Fluorescence Cell Barcoding Reaction

Note: Ensure that stock solutions of High, Medium, and Low Cell Barcoding Dyes have been prepared and thawed to ambient room temperature in advance. See Reagent Preparation section above and Table 1.

13. While cells are being permeabilized, prepare the barcoding dye solutions and labeling grid.
   a. Ensure the vials containing barcoding dye solutions are equilibrated to ambient temperature before opening them to avoid condensation and the addition of water to the dye solutions. The barcoding dyes may become unstable during prolonged exposure to aqueous solutions.
   b. Minimize exposure of the dye solutions to light.

14. Aliquot dilutions of the barcoding dyes according to the CBD Dye Dilution 96 Well Plate as shown in Figure 1.

15. Prepare a 4 × 4 Fluorescent Cell Barcoding grid pattern and CBD450 H and CBD500 H compensation cell controls in the Fluorescent Cell Barcoding Deep Well Plate (Figure 1).

16. Using a multichannel pipet, transfer 500 μL of cells suspended in 50% Perm Buffer III (step 12 above) from the Cell Preparation Plate to the Fluorescent Cell Barcoding Plate according to the Workflow Overview (Figure 1).

17. Incubate the plate at 4°C (in a refrigerator) for 30 min.

Note: This incubation time is critical for consistent cell labeling.

E. Stop the Fluorescent Cell Barcoding Reaction and wash out free dyes

18. Add 1 mL room temperature 1× Barcoding Wash Buffer to each well and centrifuge the cells at 600 g for 5 min.

19. Remove supernatants by aspirating with a manifold. Vortex the plate vigorously to loosen cell pellets.

20. Add 1 mL of the 1× Barcoding Wash Buffer to each well.
21. Centrifuge the cells at 600 g for 5 min.
22. Remove supernatants by aspirating with a manifold. Vortex the plate vigorously to loosen cell pellets.
23. Repeat wash steps 20 to 22. Vortex the plate vigorously to loosen cell pellets.

**F. Cell staining with fluorescent antibodies**

24. Resuspend cells in rows A, B, C and D in 500 μL 1× Barcoding Wash Buffer.
25. Resuspend cells in row E in 100 μL of 1× Barcoding Wash Buffer. Cells from these wells can be used for fluorescence compensation controls.
26. Combine 100 μL of cell suspension from wells in the 4 × 4 Fluorescent Cell Barcoding grid (A1 through D4 ) into a reservoir containing 2 mL 1× Barcoding Wash Buffer. Mix the reservoir contents and transfer contents to a 12 × 75 mm FACS tube labeled “Combined.” Do NOT add cells from any other wells into this tube.
   *Note:* Additional barcoded cells can be combined if multiple antibody panels are required for staining. Up to 5 separate antibody labelings can be prepared from each barcoded plate.
27. Centrifuge the cells at 600g for 5 min. Remove supernatants by decanting followed by blotting or aspirating to remove the supernatant.
28. Resuspend the cell pellet in 100 μL of 1× Barcoding Wash Buffer. Add the appropriate fluorescent antibody conjugates to the tube and incubate for 60 minutes at room temperature protected from light.

**G. Prepare compensation tubes and acquire data by multiparameter flow cytometric analysis**

29. a. Use 500 μL of cells from wells A5 and B5 for fluorescence compensation of barcoding dyes CBD450 and CBD500. Transfer cells to tubes a and b (see example below).
   b. Use 500 μL of cells from C5 or D5 for unstained fluorescence compensation controls. Transfer cells to tube c.
   c. Use 100 μL of cells from wells in Row E for fluorescence compensation. Transfer cells to tubes d, e, f, and g.
   For example:
   • Tube a. CBD450
   • Tube b. CBD500
   • Tube c. Unstained
   • Tube d. Alexa Fluor® 488
   • Tube e. PE
   • Tube f. PerCP-Cy™5.5
   • Tube g. Alexa Fluor® 647
30. Add each of the following fluorescent antibodies separately to the correspondingly labeled single color compensation control tubes (tubes d through g above). Incubate for 45 to 60 minutes at room temperature protected from light:
   • CD8 (RPA-T8)-Alexa Fluor® 488
   • CD3 (UCHT1)-PE
   • CD4 (SK3)-PerCP-Cy™5.5
   • CD4 (RPA-T4)-Alexa Fluor® 647

**H. Wash stained cells**

31. Add 3 mL of 1× Barcoding Wash Buffer to all tubes containing cells stained with fluorescent antibodies (step 28 and step 30, excluding tubes a, b, and c from step 29 above).
32. Centrifuge the cells at 600g for 5 min.
33. Remove supernatants by decanting or by aspiration.
34. Resuspend the compensation control cells in 500 μL of 1× Barcoding Wash Buffer.
35. Resuspend the combined barcoded cells in 1,000 to 1,500 μL of 1× Barcoding Wash Buffer.
36. After appropriate cytometer setup, acquire 300,000 to 500,000 events for the combined sample using either a BD FACSCanto™ II, BD LSRFortessa™ or BD™ LSR II Flow Cytometer System.

**General recommendations for cytometer set up and gating strategy**

1. Ensure that the unstained cell sample and the brightest stained cell samples are on scale for each fluorescence channel in use. Most frequently, optimal set-up of CBD450 and CBD500 requires PMT voltage settings that place unstained cells in the first decade. Application-specific settings are recommended.
2. Gating: Display CBD450 versus CBD500 fluorescence gated on the lymphocyte population. See Figure 2 below.
Fresh whole blood was obtained from a human donor and incubated for 15 min under 16 different stimulation conditions, including unstimulated (no activator) or stimulated with different doses of PMA or recombinant human IFN-α, IL-6, IL-2, or IL-4. Erythrocytes were lysed and leukocytes fixed using 1× BD Phosflow™ Lyse/Fix Buffer (Cat. No. 558049). The cells were then permeabilized using BD Phosflow™ Perm Buffer III (Cat. No. 558050), and barcoded using the BD Phosflow™ Violet Fluorescent Cell Barcoding Kit (Cat No. 561570). Barcoded samples were pooled and stained using PE Mouse Anti-Human CD3 (Cat. No. 555333), PerCP-Cy™5.5 Mouse anti-Human CD4 (Cat. No. 341654), Alexa Fluor® 488 Mouse Anti-Human CD8 (Cat. No. 557704) and Alexa Fluor® 647 Mouse Anti-Mouse Stat6 (pY641) (Cat. No. 612601). Lymphocytes were gated from the forward- versus side-light scatter plot. Subsequent cell populations of varying CD450 and CD500 labeling intensities (corresponding to one of 16 stimulation conditions) were gated as shown. Phosphorylated STAT6 expression levels were then displayed for CD3+CD4+ T cells that were either Unstimulated (red line histogram) or Stimulated with IL-4 (Cat. No. 554605) (100 ng/mL final concentration; blue line histogram) as shown.

Fresh whole blood was either left unstimulated or treated with various stimuli (16 different stimulation conditions total), then fixed, permeabilized, and barcoded as described in the Figure 2 legend above. Cells (500 µL) from each barcoded well were pooled, mixed, and split equally into six 12 × 75 mm FACS tubes for staining with Antibody Panels 1-6: PE Mouse Anti-Human CD3 (Cat. No. 555333), PerCP-Cy™5.5 Mouse Anti-Human CD4 (Cat. No. 341654), Alexa Fluor® 488 Mouse Anti-Human CD8 (Cat. No. 557704) and Alexa Fluor® 647-conjugated antibodies specific for either phosphorylated ERK1/2 (Panel 1), Stat1 (2), Stat3 (3), Stat5 (4), Stat6 (5), or p38 MAPK (6). Flow cytometric analysis was performed using a BD FACSCanto™ II Flow Cytometer. CD3+CD4+ or CD3+CD8+ T cells from 16 different stimulation conditions were identified using Cytobank™ software based on forward and side light-scatter characteristics, CD marker staining, and varying CD450 and CD500 labeling intensities, as in Figure 2. Histogram overlays (Figure 3A) show activation profiles for six phosphoproteins in CD3+CD4+ T cells from 16 different stimulation conditions (96 histograms total). Heatmap displays (Figure 3B) show activation profiles for six phosphoproteins (stained with Antibody Panels 1-6 as in Figure 3A) in CD3+CD4+ or CD3+CD8+ T cells. The color spectrum heatmap key (color scale) indicates fold change of Median Fluorescence Intensity (MFI) of Stimulated cells divided by the MFI for Unstimulated Cells (ie, yellow reflects high induction of phosphorylated signaling molecule expression whereas black reflects no induction).
Product Notices

1. The Alexa Fluor®, Pacific Blue™, and Cascade Blue® dye antibody conjugates in this product are sold under license from Molecular Probes, Inc. for research use only, excluding use in combination with microarrays, or as analyte specific reagents. The Alexa Fluor® dyes (except for Alexa Fluor® 430), Pacific Blue™ dye, and Cascade Blue® dye are covered by pending and issued patents.

2. Alexa Fluor® is a registered trademark of Molecular Probes, Inc., Eugene, OR.

3. Cy is a trademark of Amersham Biosciences Limited.


<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible cause</th>
<th>Recommended action</th>
</tr>
</thead>
<tbody>
<tr>
<td>No or incomplete red blood cell lysis</td>
<td>Lyse/Fix Buffer diluted in 1× PBS</td>
<td>Use distilled water to dilute the 5× Lyse/Fix Buffer to 1×.</td>
</tr>
<tr>
<td>Inadequate mixing with blood</td>
<td>Immediately after adding the Lyse/Fix Buffer to blood samples, mix thoroughly by pipetting up and down at least 3 times.</td>
<td></td>
</tr>
<tr>
<td>Inadequate incubation time</td>
<td>After mixing the Lyse/Fix Buffer with blood samples, incubate for 15 minutes in a 37°C incubator.</td>
<td></td>
</tr>
<tr>
<td>Improper whole blood to Lyse/Fix Buffer ratio</td>
<td>Use at least 10 times the volume of whole blood for the Lyse/Fix Buffer treatment step.</td>
<td></td>
</tr>
<tr>
<td>No or poor surface CD marker staining</td>
<td>Cells are exposed to Lyse/Fix Buffer too long</td>
<td>Incubation time in the Lyse/Fix Buffer should not exceed 15 minutes at 37°C.</td>
</tr>
<tr>
<td></td>
<td>Delay in centrifugation</td>
<td>After the Lyse/Fix step, the Lyse/Fix Buffer should be removed immediately by centrifuging the cells, removing the supernatant, and washing the cells once with 1× PBS.</td>
</tr>
<tr>
<td></td>
<td>Cells are exposed to Perm Buffer III for too long at temperatures greater than 4°C</td>
<td>Do not exceed the recommended permeabilization time, ie, 30 minutes on ice. When cells are in Perm Buffer III, they should be kept cold. After the fluorescence barcoding reaction in 50% Perm Buffer III, 1× Fluorescence Barcoding Wash Buffer should be added immediately, followed by immediate washes with the Barcoding Wash Buffer.</td>
</tr>
<tr>
<td>No or poor cellular activation</td>
<td>No activator/biological response modifier added</td>
<td>Add the appropriate amount of activator/biological response modifier.</td>
</tr>
<tr>
<td></td>
<td>Activator/biological response modifier too old, expired, or lost activity</td>
<td>Prepare fresh activator/biological response modifier and store as aliquots. Use new aliquots for all experiments. Avoid repeated freeze/thaw cycles.</td>
</tr>
<tr>
<td></td>
<td>No carrier protein in the dilution buffer for activators/biological response modifiers</td>
<td>Use a buffer (such as PBS or Dulbecco’s PBS) containing at least 1 mg/mL of BSA to dilute the activators/biological response modifiers, and keep the diluted stimulant on ice until use.</td>
</tr>
<tr>
<td></td>
<td>Diluted activator/biological response modifier kept too long before use</td>
<td>We recommend using activators/biological response modifiers within 30 minutes of dilution.</td>
</tr>
<tr>
<td></td>
<td>Cell populations identified on the barcoding plot do not match the wells containing activators</td>
<td>Ensure that the cells are transferred correctly from the Cell Preparation Plate to the Fluorescent Cell Barcoding Plate as shown in Figure 1 (Step 2).</td>
</tr>
<tr>
<td>Low or no cell barcoding signal</td>
<td>Barcoding dyes not added</td>
<td>Add appropriate amount of barcoding dyes to label cells.</td>
</tr>
<tr>
<td></td>
<td>Barcoding dyes lost reactivity</td>
<td>Use a new aliquot or buy new dyes. Do NOT mix different vials or batches of barcoding dyes.</td>
</tr>
<tr>
<td></td>
<td>Reaction time too short</td>
<td>The barcoding reaction time should be 30 min.</td>
</tr>
<tr>
<td></td>
<td>Wrong dilution of the dyes</td>
<td>Check the Cell Barcoding Dye dilutions.</td>
</tr>
<tr>
<td>Cell barcoding signal too high</td>
<td>Wrong dilution of the dyes</td>
<td>Check the Cell Barcoding Dye dilutions.</td>
</tr>
<tr>
<td></td>
<td>Reaction time too long</td>
<td>The barcoding reaction time should be no longer than 35 min.</td>
</tr>
<tr>
<td>Rare Events</td>
<td>Too few event acquired</td>
<td>Consider the frequency of the intended target cell population(s) to determine the required total number of acquired events necessary to ensure statistical significance (Parks, 1989)</td>
</tr>
</tbody>
</table>

Table 3. Troubleshooting

References


